

Interfamilial characterization of a region of the ZFX and ZFY genes facilitates sex determination in cetaceans and other mammals

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Abstract

Sequence polymorphism of homologues ZFX and ZFY, in a 604-base pair exon region, was examined in 10 known males and 10 known females across seven cetacean families and used to design a simple, highly sensitive and widely applicable fluorescent 5' exonuclease assay for gender determination in cetaceans. Multiplex amplification, cloning, and sequencing of these previously uncharacterized regions revealed (i) eight fixed differences between ZFX and ZFY, (ii) 29 variable sites between ZFX and ZFY and (iii) very low interspecific nucleotide diversity for both ZFX and ZFY across all families examined. We developed a 5' exonuclease assay that produces a small (105 bp) polymerase chain reaction (PCR) product from both the X and the Y chromosome orthologs, and used double-labelled fluorescent probes to distinguish between the two genes in a real-time PCR assay that is highly reproducible and sensitive. We demonstrated sex specificity for 33 cetacean species in nine families. Given the availability of conserved primers and sequence information for many mammalian species, this approach to designing sexing assays for a wide range of species is both practical and efficient.

Keywords: 5' exonuclease assay, cetacean, gender, sex determination, SNP, TaqMan, ZFX, ZFY

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Introduction

Sexing of cetaceans is critical for population genetic, behavioural and demographic studies, but difficult in the field because of limited sexual dimorphism in many species and internal genitalia. Genetic assays for sexing have been developed, and typically included co-amplification of Y chromosome and autosomal or X chromosome targets, followed by separation of different-sized fragments on agarose gels, or restriction digestion of like-sized fragments followed by gel electrophoresis and visualization. To date, these assays have been useful on only a subset of the Cetacea, and/or have proved unreliable for degraded DNA.

SRY (sex-determining Y chromosome gene) detection via polymerase chain reaction (PCR) has become a widely used gender determination method across a variety of mammalian species (Taberlet *et al.* 1993; Richard *et al.* 1994; Finch *et al.* 1996; Takahashi *et al.* 1998; Wilson & White 1998; Rosel 2003). Lack of SRY amplification, however, is not solely caused by gene absence (Palsbøll *et al.* 1992; Taberlet *et al.* 1993) and may reflect lack of PCR amplification. To clarify the equivocal nature of negative results, some methods rely on an internal positive control (Taberlet *et al.* 1993; Takahashi *et al.* 1998; Wilson & White 1998) to indicate successful PCR amplification, or co-amplify orthologous genes such as amelogenin (ameloX/Y, Nakahori *et al.* 1991; Morin *et al.* 1999) or ZFX/ZFY (Aasen & Medrano 1990; Palsbøll *et al.* 1992; Bérubé & Palsbøll 1996; Reynolds & Varlaro 1996), but differences in the sizes or efficiency of amplification of the products can still lead to incorrect inference of sex, especially when DNA quantity or quality is low (Rosel 2003).

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Because small-sized DNA fragment resolution in agarose is limited, PCR product sizes have tended to be in the range of 300 to 800 bp. If DNA samples are degraded or of very low concentration, larger fragments can amplify poorly or not at all, limiting the accuracy of the assays for these samples. Marine mammal specimens for use in molecular ecology and population structure studies are commonly obtained from three primary sources: (i) strand-ing events, where specimens are often at decomposition levels that prevent accurate assessment of reproductive organs, (ii) forensic samples obtained from international markets, and (iii) directed biopsy from live animals at sea. More recently, 'ancient DNA' studies have begun to make use of bone and tooth samples from museum specimens and other skeletal remains (Rosenbaum *et al.* 1997; Rastogi *et al.* 2004). For these samples, which often yield low quality and quantity DNA, a sexing assay is needed that is highly sensitive for both the X and Y chromosomes, uses one pair of PCR primers for amplification of orthologous loci, and which produces a small PCR product size. Ideally, the product should be as small as can be amplified and detected by PCR, on the order of 70–150 bp, to maximize the efficiency of amplification from degraded or low quantity samples (Smith *et al.* 2002).

The 5' exonuclease assay (Holland *et al.* 1991; Livak *et al.* 1995a; Morin *et al.* 1999) is a PCR-based assay, which incorporates double-labelled fluorescent oligonucleotide probes which hybridize to the template DNA during the PCR, and are cleaved by the exonuclease activity of *Taq* as the template is replicated, releasing the reporter dye from proximity to the quencher, and producing fluorescent signal in direct proportion to the number of copies of the template formed during PCR. Multiple sequence-specific probes labelled with different fluorescent reporter dyes can be used to detect sequences differing by as little as one nucleotide [e.g. for single nucleotide polymorphisms (SNPs) or

orthologous genes], and assays are often sensitive over several orders of magnitude of DNA concentration (Morin *et al.* 1999, 2004).

The last exon of ZFX/ZFY, located most proximal to the centromere of four conserved segments and encoding 13 zinc-finger domains, is highly homologous among placental mammals, where ZFX has been shown to escape X-inactivation (Page *et al.* 1987; Schneider-Gadicke *et al.* 1989; Jegalian & Page 1998). The present study determines the ZFX and ZFY sequences from nucleotides 96 to 699 (human sequence, Schneider-Gadicke *et al.* 1989) across seven cetacean families, identifies fixed differences between ZFX and ZFY in these families, and presents a new fluorescent 5' exonuclease assay which identifies the orthologous ZFX and ZFY PCR products in a simple closed-tube assay for rapid, efficient, and highly sensitive sexing of cetaceans. Comparison of these sequences to published sequences from other mammalian orders indicates high sequence conservation and patterns of X/Y differences that should allow similar assay development for other groups of species and families.

Materials and methods

ZFX/ZFY sequencing

Ten cetacean species from seven families were used for ZFX and ZFY sequencing. One known male and one known female from eight odontocete and two mysticete species were selected from the Southwest Fisheries Science Center (SWFSC) tissue archive (Table 1). Sex was determined by physical examination of the reproductive organs, except in the case of *Physeter macrocephalus* where determination was made by external examination of the genital slits.

Whole genomic DNA from all tissue samples used for DNA sequencing was obtained using one of four extraction

Table 1 Eight odontocete and two mysticete species examined from seven families, code abbreviations, and GenBank Accession nos

Family	Species	Common name	Code	GenBank Accession no.		
				ZFX (f)	ZFX (m)	ZFY
Balaenidae	<i>Balaena mysticetus</i>	Bowhead whale	BW	AF260783	AF260784	AF260785
Eschrichtiidae	<i>Eschrichtius robustus</i>	Grey whale	GW	AF260789	AF260790	AF260791
Physeteridae	<i>Physeter macrocephalus</i>	Sperm whale	SW	AF260801	AF260802	AF260803
Kogiidae	<i>Kogia breviceps</i>	Pygmy sperm whale	PS	AF260792	AF260793	AF260794
	<i>Kogia sima</i>	Dwarf sperm whale	DS	AF260797	AF260795	AF260796
Monodontidae	<i>Delphinapterus leucas</i>	Beluga	BL	AF260788	AF260786	AF260787
Delphinidae	<i>Stenella longirostris orientalis</i>	Eastern spinner dolphin	ES	AF260812	AF260810	AF260811
	<i>Stenella longirostris hybrid</i>	Whitebelly spinner dolphin*	WS	AF260807	AF260808	AF260809
Phocoenidae	<i>Phocoena phocoena</i>	Harbour porpoise	HP	AF260804	AF260805	AF260806
	<i>Neophocaena phocaenoides</i>	Finless porpoise	FP	AF260800	AF260798	AF260799

*Intergrade form or hybrid between *Stenella longirostris longirostris* and *Stenella longirostris orientalis*. The subspecies status is not yet defined (Rice 1998).

methods: (i) FastDNA kit (BIO101, Inc.), (ii) hexadecyltrimethylammonium bromide (CTAB) (modified from Winnepeninckx *et al.* 1993), (iii) 25:24:1 phenol–chloroform–isoamyl alcohol followed by ethanol precipitation (modified from Sambrook *et al.* 1989), or (iv) lithium chloride (Gemmell & Akiyama 1996). All 20 DNA extracts were quantified on a TD700 Fluorometer (Turner Designs) using Picogreen dsDNA Quantification Reagent (Molecular Probes).

Multiplex amplification of ZFX and ZFY, along with a negative control, was accomplished via a 50- μ L PCR containing *Pfu* 1 \times Reaction Buffer (Stratagene), 0.2 μ M each of forward primer ZFY0097 (CATCCTTTGACTGTCTATCCTTG, Palsbøll *et al.* 1992) and reverse primer P2-3EZ (GCACTTCTTTGGTATCTGAGAAAGT, Aasen & Medrano 1990), 100 μ M each dNTP, 1.25 units cloned *Pfu* DNA polymerase (Stratagene), and 4–164 ng genomic DNA. With a mineral oil overlay, cycling was performed on a Perkin-Elmer Cetus DNA Thermal Cycler (TC1) with the following parameters: denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 48 °C for 45 s, extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. The ZFX and ZFY amplicons produced are both 604 bp and appear as one overlapping fragment on an agarose gel.

PCR products were cloned to obtain sequences of ZFX and ZFY. *Pfu* DNA polymerase amplicons do not possess nontemplated 3' A overhangs, so PCR products were incubated with 2.5 units *Taq* DNA polymerase (Gibco BRL Life Technologies) at 72 °C for 10 min to generate the nontemplated 3' A nucleotide addition. These amplicons were purified with the QIAquick PCR Purification Kit (QIAGEN). Using the TOPO TA Cloning Kit, clean PCR product was ligated with pCR2.1-TOPO plasmid vector and transformed into TOP10 One Shot Cells (Invitrogen). One microlitre of plasmid DNA, isolated with Ultraclean Mini Plasmid Prep Kit (Mo Bio Laboratories, Inc.), was digested with 4 units *Eco*RI (New England BioLabs) at 37 °C for 1 h. Reactions were stopped with 2 μ L bromophenol blue Type IV buffer (Sambrook *et al.* 1989) and the full volume electrophoresed on 1% agarose minigels to verify PCR product insertion.

Both strands of isolated plasmids were directly sequenced in a 15- μ L volume using 3 μ L of ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), 8 μ L of 1 μ M vector specific primers and 1–4 μ L plasmid DNA according to the TOPO TA Cloning Instruction Manual. Unincorporated dye terminators were removed with an ethanol/sodium acetate precipitation modified from the BigDye Terminator Cycle Sequencing Ready Reaction Kit User Protocol. Sequences were electrophoresed on an Applied Biosystems ABI 377 automated DNA sequencer using a 4.5% polyacrylamide gel. Sequences were aligned, vector DNA excised, and edited with SEQUENCHER version 3.1.1 (Gene Codes Corporation).

5' exonuclease assay for allelic discrimination

Samples used to validate the 5' exonuclease assay were obtained from the SWFSC tissue archive, and included at least one animal of each sex from 33 species in nine cetacean families (Table 2). Whenever possible, samples were selected which had been sexed by visual inspection of genitalia or gonads, but this was not always possible, so the sex of some samples was corroborated by other methods (e.g. an alternative sexing assay based on co-amplification and electrophoresis of SRY and ZFX PCR products (Fain & Lemay 1995) (Table 2).

The 5' exonuclease assay targeted a 105-bp region of the ZFX and ZFY aligned gene sequences, including two fixed differences at nucleotides 179 and 191. Primers and probes were designed using published guidelines (Livak *et al.* 1995b; Morin *et al.* 1999). The primers were designed to anneal at a predicted 59 °C and to closely flank an oligonucleotide probe annealing at a predicted 7 °C above the annealing temperature (T_a) of the primers. The primers and probes were selected using the software program PRIMER EXPRESS (Applied Biosystems). Probes were synthesized with a 5' reporter dye (CY5 for ZFY, FAM for ZFX) and a 3' quencher (Iowa Black FQ or RQ; Integrated DNA Technologies, Inc.). The oligonucleotide sequences were forward primer (CetZFX_F1) AGTTTAAGTCGAGAGGTTTTTTGAAA, reverse primer (CetZFX_R1) TCTTGTTGGTAGTGTAAATCACAGTCAGT, and probes CETZFXprobe Fam-AAAACCATCCTGAACACCTTACCAAGAA-IowaBlackFQ, CETZFYprobe Cy5-AACCACCTGAACACCTCACCAA-IowaBlackRQ. The ZFX probe has two mismatches to the human ZFX sequence, and one mismatch with the human ZFY sequence. The ZFY probe has three mismatches to the human ZFY sequence, and four mismatches to the human ZFX sequence. The 5' exonuclease assay was performed in 25- μ L PCRs containing 1 \times PCR buffer (SureStart *Taq*, Stratagene, or HotStarTaq, QIAGEN), 6 mM $MgCl_2$, 150 μ M each dNTP, 0.05 U/ μ L SureStart or HotStarTaq polymerase, 600 nM each primer, 100 nM each probe, 0.4 μ g/ μ L BSA (bovine serum albumin) and approximately 20–40 ng DNA. PCR amplification was performed in a MX3000P Real-Time PCR System (Stratagene), with initial incubations for 10–15 min at 94 °C, followed by either 40 cycles of 94 °C for 45 s and 56 °C for 45 s, or 40 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s (both programmes work equally well, but the 2-step PCR takes less time).

Results

Sequencing of cloned ZFX and ZFY products revealed a single 604-bp sequence from each female (XX) and two sequences from each male (XY) from each of the 10 species. A majority-rule consensus sequence for all clones was

Table 2 Samples screened with the 5' exonuclease assay to determine sex. The method column lists the sexing method used to originally sex the individual, or to confirm sex with a second method in some cases. 'SRY assay' refers to the method of Fain & Lemay (1995)

Family	Species	Common name	Laboratory ID	Sex	Method
Balaenidae	<i>Balaena mysticetus</i>	Bowhead whale	6980	F	unknown
	<i>Balaena mysticetus</i>	Bowhead whale	6981	M	unknown
	<i>Eubalaena japonica</i>	North Pacific right whale	28424	F	SRY assay
	<i>Eubalaena japonica</i>	North Pacific right whale	28425	M	SRY assay
Balaenopteridae	<i>Balaenoptera acutorostrata</i>	Minke whale	2161	M	gonads
	<i>Balaenoptera acutorostrata</i>	Minke whale	23633	F	unknown
	<i>Balaenoptera borealis</i>	Sei whale	3992	F	unknown
	<i>Balaenoptera borealis</i>	Sei whale	3994	F	unknown
	<i>Balaenoptera borealis</i>	Sei whale	3999	M	unknown
	<i>Balaenoptera edeni</i>	Bryde's whale	7643	F	unknown
	<i>Balaenoptera edeni</i>	Bryde's whale	32258	M	gonads
	<i>Balaenoptera musculus</i>	Blue whale	25437	F	SRY assay
	<i>Balaenoptera musculus</i>	Blue whale	29847	M	unknown, SRY failed
	<i>Balaenoptera physalus</i>	Fin whale	2821	M	external exam
	<i>Balaenoptera physalus</i>	Fin whale	10743	F	unknown
	<i>Megaptera novaeangliae</i>	Humpback whale	2813	M	external exam
	<i>Megaptera novaeangliae</i>	Humpback whale	28499	F	SRY assay
Delphinidae	<i>Delphinus capensis</i>	Long-beaked common dolphin	28947	M	gonads
	<i>Delphinus capensis</i>	Long-beaked common dolphin	32265	F	unknown
	<i>Delphinus delphis</i>	Short-beaked common dolphin	32259	F	gonads
	<i>Delphinus delphis</i>	Short-beaked common dolphin	32260	M	gonads
	<i>Globicephala macrorhynchus</i>	Short-finned pilot whale	1685	F	external exam
	<i>Globicephala macrorhynchus</i>	Short-finned pilot whale	2819	M	gonads
	<i>Globicephala melas</i>	Long-finned pilot whale	463	M	external exam
	<i>Globicephala melas</i>	Long-finned pilot whale	469	M	external exam
	<i>Globicephala melas</i>	Long-finned pilot whale	1405	F	gonads
	<i>Globicephala melas</i>	Long-finned pilot whale	1406	F	gonads
	<i>Grampus griseus</i>	Risso's dolphin	141	F	external exam
	<i>Grampus griseus</i>	Risso's dolphin	39084	M	external exam
	<i>Lagenodelphis hosei</i>	Fraser's dolphin	394	M	unknown
	<i>Lagenodelphis hosei</i>	Fraser's dolphin	2509	F	external exam
	<i>Lagenodelphis hosei</i>	Fraser's dolphin	2595	F	external exam
	<i>Lagenorhynchus albirostris</i>	White-beaked dolphin	17311	M	unknown
	<i>Lagenorhynchus albirostris</i>	White-beaked dolphin	17318	F	unknown
	<i>Lagenorhynchus albirostris</i>	White-beaked dolphin	17319	F	unknown
	<i>Lagenorhynchus albirostris</i>	White-beaked dolphin	23523	F	unknown
	<i>Lagenorhynchus obliquidens</i>	Pacific white-sided dolphin	741	F	gonads
	<i>Lagenorhynchus obliquidens</i>	Pacific white-sided dolphin	3724	M	gonads
	<i>Lagenorhynchus obliquidens</i>	Pacific white-sided dolphin	4817	F	unknown
	<i>Lissodelphis borealis</i>	Northern right whale dolphin	9217	M	SRY assay
	<i>Lissodelphis borealis</i>	Northern right whale dolphin	23163	F	unknown
	<i>Orcinus orca</i>	Killer whale	26565	F	external exam
	<i>Orcinus orca</i>	Killer whale	26567	M	unknown
	<i>Orcinus orca</i>	Killer whale	26568	F	unknown
	<i>Pseudorca crassidens</i>	False killer whale	27456	F	SRY assay
	<i>Pseudorca crassidens</i>	False killer whale	27510	M	gonads
	<i>Stenella attenuata</i>	Pantropical spotted dolphin	2084	F	external exam
	<i>Stenella attenuata</i>	Pantropical spotted dolphin	2085	M	external exam
	<i>Stenella coeruleoalba</i>	Striped dolphin	875	M	gonads
	<i>Stenella coeruleoalba</i>	Striped dolphin	876	F	gonads
	<i>Steno bredanensis</i>	Rough-toothed dolphin	138	M	gonads
	<i>Steno bredanensis</i>	Rough-toothed dolphin	139	F	gonads
	<i>Steno bredanensis</i>	Rough-toothed dolphin	459	M	external exam
	<i>Steno bredanensis</i>	Rough-toothed dolphin	461	M	external exam
	<i>Steno bredanensis</i>	Rough-toothed dolphin	544	F	external exam
	<i>Tursiops truncatus</i>	Bottlenose dolphin	4363	M	unknown
	<i>Tursiops truncatus</i>	Bottlenose dolphin	4366	F	unknown

Table 2 Continued

Family	Species	Common name	Laboratory ID	Sex	Method
Eschrichtiidae	<i>Eschrichtius robustus</i>	Gray whale	856	M	external exam
	<i>Eschrichtius robustus</i>	Gray whale	23327	F	SRY assay
Kogiidae	<i>Kogia breviceps</i>	Pygmy sperm whale	10117	F	unknown
	<i>Kogia breviceps</i>	Pygmy sperm whale	10119	M	unknown
	<i>Kogia sima</i>	Dwarf sperm whale	10124	F	unknown
	<i>Kogia sima</i>	Dwarf sperm whale	17104	F	unknown
	<i>Kogia sima</i>	Dwarf sperm whale	23604	M	unknown
	<i>Kogia sima</i>	Dwarf sperm whale	26683	F	external exam
Monodontidae	<i>Delphinapterus leucas</i>	Beluga or White whale	28926	M	external exam
	<i>Delphinapterus leucas</i>	Beluga or White whale	28926	M	external exam
	<i>Monodon monocerus</i>	Narwhal	8229	F	unknown
	<i>Monodon monocerus</i>	Narwhal	8230	F	unknown
	<i>Monodon monocerus</i>	Narwhal	8243	M	unknown
	<i>Monodon monocerus</i>	Narwhal	8244	F	unknown
Phocoenidae	<i>Phocoena phocoena</i>	Harbor porpoise	1084	F	gonads
	<i>Phocoena phocoena</i>	Harbor porpoise	1353	F	gonads
	<i>Phocoena phocoena</i>	Harbor porpoise	26606	M	unknown
	<i>Phocoenoides dalli</i>	Dall's porpoise	713	M	gonads
	<i>Phocoenoides dalli</i>	Dall's porpoise	1880	F	gonads
	<i>Phocoenoides dalli</i>	Dall's porpoise	3735	F	gonads
	<i>Phocoenoides dalli</i>	Dall's porpoise	3739	M	gonads
	<i>Phocoenoides dalli</i>	Dall's porpoise	3739	M	gonads
Physeteridae	<i>Physeter macrocephalus</i>	Sperm whale	2364	F	external exam
	<i>Physeter macrocephalus</i>	Sperm whale	2365	F	external exam
	<i>Physeter macrocephalus</i>	Sperm whale	9698	F	external exam
	<i>Physeter macrocephalus</i>	Sperm whale	9708	M	external exam
Ziphiidae	<i>Mesoplodon densirostris</i>	Blaineville's beaked whale	8681	M	unknown
	<i>Mesoplodon densirostris</i>	Blaineville's beaked whale	27415	F	SRY assay
	<i>Ziphius cavirostris</i>	Cuvier's beaked whale	745	F	SRY assay
	<i>Ziphius cavirostris</i>	Cuvier's beaked whale	1120	M	gonads
	<i>Ziphius cavirostris</i>	Cuvier's beaked whale	30071	M	SRY assay

generated for each of these sequences for all samples. The resultant 30 consensus sequences were subject to 1000 bootstrap replicates in a full heuristic search based on maximum parsimony using PAUP version 4.0b4a (Swofford 1999), with human ZFX and ZFY as outgroups. The search results produced two distinct clades (Fig. 1). The separation of cetacean from human alleles was supported by a confidence value of 99. All sequences attributed to females plus half the sequences attributed to males formed one clade, ZFX, supported by a confidence value of 99. The remaining 10 male consensus sequences formed the other clade, ZFY, supported by a confidence value of 96.

Six hundred four base pairs of ZFX/ZFY open reading frame (ORF) were sequenced (Fig. 2). Comparison of the ZFX ortholog obtained from males with the same ortholog obtained from females revealed nine variable sites, of which sites 335 and 515 were only detected in the males of *Kogia breviceps* and *Stenella longirostris orientalis/longirostris* hybrid, respectively (Fig. 3). A total of eight different ZFX haplotypes were observed in the 20 sequences. All the ZFX variable sites displayed synonymous substitutions result-

ing in a lack of amino acid heterozygosity across all the families examined (Fig. 4).

The 10 ZFY sequences displayed 22 variable sites resulting in eight different haplotypes (Fig. 3). Seven of the variable nucleotide sites produced six amino acid variable positions resulting in five amino acid haplotypes (Figs 3 and 4), all of which maintain the ORF. Kogiidae exhibited a 3-bp deletion at 379–381 bp on the ZFY, causing concatenation of nucleotides in codons 95 (Gly) and 96 (Ala) into one codon for alanine (Ala); this did not affect the ORF.

Comparison of ZFX and ZFY from all species revealed eight fixed differences, all synonymous, and 29 variable sites, of which two were variable on both ZFX and ZFY (Fig. 3). The fixed difference at position 302 forms a *TaqI* restriction site in ZFX but not in ZFY. An additional *TaqI* restriction location (denoted by the TCGA sequence) was found for both ZFX and ZFY beginning at position 144. No variability was detected at any of the restriction sites. The restriction pattern for a ZFX fragment exhibits sizes 49, 157, and 399. Likewise, a ZFY fragment exhibits sizes 49 and

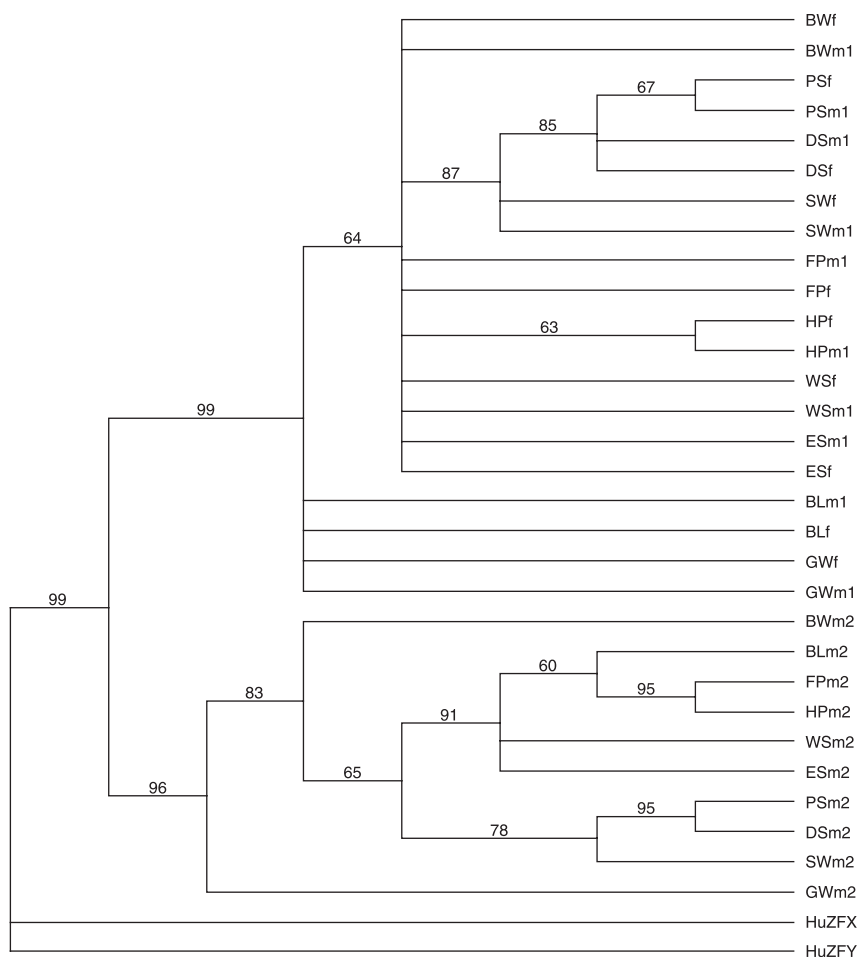


Fig. 1 The 50% majority-rule consensus tree of the 30 cetacean sequences following 1000 bootstrap replicates and rooted with outgroups HuZFX (human ZFX; Schneider-Gadicke *et al.* 1989) and HuZFY (human ZFY; Page *et al.* 1987). f, female; m, male. 1, ZFX; 2, ZFY. All characters are unordered and of equal weight. The starting tree was obtained via stepwise addition. Sequence addition followed the 'simple' algorithm using BWf as the reference taxon. Steepest descent and the tree-bisection-reconnection (TBR) branch swapping algorithm were in effect. Species codes are as in Table 1.

556. Therefore, females (XX) display the fragments 49, 157, and 399 whereas males (XY) display the fragments 49, 157, 399, and 556. All fragments are displayed in equal intensity on a minigel.

The high haplotypic diversity observed in both ZFX and ZFY results from very low nucleotide diversity. A pairwise uncorrected 'p' distance matrix of all ZFX haplotypes estimated nucleotide diversity between 0.2–1.2% with a mean equal to 0.6%. A similar calculation for all ZFY haplotypes estimated nucleotide diversity between 0.2–2.3% with a mean equal to 1.4%. The above two calculations were repeated with the inclusion of the human ZFX and human ZFY, respectively. The human ZFX was different from the cetacean ZFX by 3.3–4.0% with a mean equal to 3.6%. The human ZFY was different from the cetacean ZFY by 5.5–6.3% with a mean equal to 6.0%. All distance calculations were computed in PAUP (Swofford 1999).

Neither ZFX nor ZFY provides strong phylogenetic signal for the species sequenced, though there is strong support for the monophyly of the Kogiidae and Physeteridae (pygmy and dwarf sperm whales and sperm whale) at both loci, and ZFY clusters the Delphinidae together as mono-

phyletic group with strong support (Fig. 1). Studies of ZFX and ZFY in the Felidae (Pecan Slattery *et al.* 2000) and some artiodactyls (Poloumienko 2004) and primates (Kim & Takenaka 2000) have previously shown that these genes do not provide strong phylogenetic signal within families. Within Felidae, there have been two ancestral episodes of gene conversion from X to Y (Pecan Slattery *et al.* 2000). Phylogenetic analysis of the cetacean data does not indicate substantial gene conversion within the family. Analysis of variable nucleotide sites (Fig. 3) among species shows some ZFY sites that are variable among species, and tend to cluster in related species, but it is not clear with these few species whether these represent instances of gene conversion over smaller portions of the gene, or just patterns of derived nucleotides in some lineages.

The ZFX and ZFY specificity of the 5' exonuclease assay is shown in Fig. 5. The Cy5 fluorescent dye is less stable than FAM to repeated freezing and thawing, and exposure to light, and may produce ambiguous results if degraded. Fortunately, this is usually easy to identify if a female control is run with each assay, as the CY5 (ZFY) signal (which is normally close to zero in females) will increase as the

	ZFY0097	CETZFX F1
Bowhead ZFX	<u>CATCCTTTGACTGTCTATCCTTG</u> CATGATTGTGGGAAAAGTTTAAGTCGAGAGGTTT	
Human ZFXG.....	
<i>S. boliviensis</i> ZFXG.....	
<i>P. cynocephalus</i> ZFXG.....	
<i>Felis chaus</i> ZFXC.....A.....C.....	
<i>Bos taurus</i> ZFXC.....	
Bowhead ZFYT.....G.....	
Human ZFYG.....G.....	
<i>S. boliviensis</i> ZFYT.....T.....G.....A.....	
<i>P. cynocephalus</i> ZFYG.....	
<i>Felis chaus</i> ZFYC.....A.....A.....A.....	
<i>Bos taurus</i> ZFYC.....T.....	
BOWHEAD PROTEIN	H P L T V Y P C M I C G K K F K S R G F	20
	CetZFXprobe & CetZFYprobe	
Bowhead ZFX	<u>TTGAAAAGGCACATGAAAACCATCCTGAACACCTTACCAAGAAGATACCGCTGTACT</u>	
Human ZFXC.....G.....A.....	
<i>S. boliviensis</i> ZFXC.....G.....	
<i>P. cynocephalus</i> ZFXC.....G.....A.....	
<i>Felis chaus</i> ZFXC.....	
<i>Bos taurus</i> ZFXC.....	
Bowhead ZFYC.....C.....	
Human ZFYA.....G.....A.....	
<i>S. boliviensis</i> ZFYA.....T.....G.....A.....	
<i>P. cynocephalus</i> ZFYA.....C.....G.....A.....	
<i>Felis chaus</i> ZFYC.....A.....A.....	
<i>Bos taurus</i> ZFYT.....A.....	
BOWHEAD PROTEIN	L K R H M K N H P E H L T K K K Y R C T	40
	CetZFX R1	
Bowhead ZFX	<u>GACTGTGATTACACTACCAACAAGAAGATAAGTTTACACAACCACTGGAGGCCACAAG</u>	
Human ZFXA.....A.....	
<i>S. boliviensis</i> ZFXA.....	
<i>P. cynocephalus</i> ZFXT.....	
<i>Felis chaus</i> ZFX	
<i>Bos taurus</i> ZFX	
Bowhead ZFY	
Human ZFYT.....T.....	
<i>S. boliviensis</i> ZFYT.....T.....	
<i>P. cynocephalus</i> ZFYT.....T.....	
<i>Felis chaus</i> ZFYT.....T.....	
<i>Bos taurus</i> ZFYT.....T.....	
BOWHEAD PROTEIN	D C D Y T T N K K I S L H N H L E S H K	60
Bowhead ZFX	CTGACCAGCAAGGCAGAGAAGGCCATCGAATGCGAGAAATGTGGGAAGCATTCTCTCAC	
Human ZFXT.....T.....G.....T.....	
<i>S. boliviensis</i> ZFXT.....G.....	
<i>P. cynocephalus</i> ZFXA.....T.....G.....T.....	
<i>Felis chaus</i> ZFXC.....T.....G.....	
<i>Bos taurus</i> ZFXT.....G.....T.....G.....C.....A.....T.....	
Bowhead ZFYG.....T.....T.....G.....T.....T.....	
Human ZFYT.....T.....T.....G.....T.....T.....	
<i>S. boliviensis</i> ZFYA.....T.....T.....T.....G.....T.....T.....	
<i>P. cynocephalus</i> ZFYT.....T.....T.....G.....T.....T.....	
<i>Felis chaus</i> ZFYA.....C.....T.....T.....G.....T.....T.....	
<i>Bos taurus</i> ZFYT.....T.....T.....T.....C.....C.....T.....	
BOWHEAD PROTEIN	L T S K A E K A I E C D E C G K H F S H	80
Bowhead ZFX	GCTGGGGCTTTGTGTTACTCACAATGGTGCATAAGGAAAAGGAGCCACAAAATGCAC	
Human ZFXA.....	
<i>S. boliviensis</i> ZFXA.....	
<i>P. cynocephalus</i> ZFXA.....	
<i>Felis chaus</i> ZFXG.....	
<i>Bos taurus</i> ZFXT.....T.....	
Bowhead ZFYT.....	
Human ZFYA.....G.....	
<i>S. boliviensis</i> ZFYA.....C.....A.....	
<i>P. cynocephalus</i> ZFYG.....C.....G.....	
<i>Felis chaus</i> ZFYC.....A.....	
<i>Bos taurus</i> ZFYC.....G.....T.....	
BOWHEAD PROTEIN	A G A L F T H K M V H K E K G A N K M H	100
Bowhead ZFX	AAGTGTAATTCGTGTAATATGAGACAGCTGAACAAGGGTGTGTAATCGCCACCTTTTG	
Human ZFXC.....A.....C.....	
<i>S. boliviensis</i> ZFXC.....G.....C.....A.....C.....	
<i>P. cynocephalus</i> ZFXC.....AC.....C.....	
<i>Felis chaus</i> ZFXC.....C.....A.....	
<i>Bos taurus</i> ZFXA.....C.....AC.....	
Bowhead ZFYC.....A.....	
Human ZFYG.....A.....C.....	
<i>S. boliviensis</i> ZFYC.....A.....	
<i>P. cynocephalus</i> ZFYG.....A.....T.....C.....	
<i>Felis chaus</i> ZFYA.....A.....A.....	
<i>Bos taurus</i> ZFYG.....A.....A.....	
BOWHEAD PROTEIN	K C K F C E Y E T A E Q G L L N R H L L	120
Bowhead ZFX	GCGGTCCACAGCAAGAACCTTCCTCATATATGTGTGGAGTGCAGTAAGGTTTTCGTGAC	
Human ZFXA.....T.....T.....G.....	
<i>S. boliviensis</i> ZFXT.....T.....G.....	
<i>P. cynocephalus</i> ZFXT.....T.....G.....	
<i>Felis chaus</i> ZFXT.....T.....	
<i>Bos taurus</i> ZFXT.....C.....T.....T.....	
Bowhead ZFY	
Human ZFYA.....T.....T.....C.....A.....	
<i>S. boliviensis</i> ZFYA.....T.....T.....C.....	
<i>P. cynocephalus</i> ZFYA.....T.....T.....C.....A.....	
<i>Felis chaus</i> ZFYA.....T.....T.....	
<i>Bos taurus</i> ZFYA.....C.....A.....T.....	
BOWHEAD PROTEIN	A V H S K N F P H I C V E C G K G F R H	140

Fig. 2 ZFX/ZFY nucleotide and amino acid consensus sequences from the bowhead whale compared to ZFX/ZFY sequences from human (Accession No. J03134, X59740), Bolivian squirrel monkey (*Saimiri boliviensis*; X75175, X75170), yellow baboon (*Papio cynocephalus*; X75174, X75173), jungle cat (*Felis chaus*; AF252990, AF253013), and cow (*Bos taurus*; NM 177490, NM 177491). Site numbering according to human ZFX (Schneider-Gadicke *et al.* 1989) and human ZFY (Page *et al.* 1987) sequences. Dots denote nucleotide agreement with bowhead ZFX. Gray highlighted amino acids indicate variable positions on the cetacean ZFY. Primer regions are underlined and names noted above. The probe sequences are shown in bold and/or outlined. *TaqI* restriction sites are double underlined. The amino acid sequence from bowhead whales is identical on the ZFX and ZFY. A comparison of all cetacean ZFY sequences to the human ZFY revealed 27 fixed differences (not shown). A comparison of all cetacean ZFX sequences to the human ZFX revealed 18 fixed differences (not shown).

Bowhead_ZFX	CCATCAGAGCTCAAAAAGCACATGCGAATCCATACTGGGGAGAAGCCGTACCAATGCCAG
Human_ZFX	.G.....A.....
S.boliviensis_ZFX	.G.....A.....
P.cynocephalus_ZFX	.C.....A.....
Felis_chaus_ZFX	.G.....G.....G.....
Bos_taurus_ZFXC.....G.....
Bowhead_ZFY	.G.....
Human_ZFY	.G..G..A..G..G.....C..C.....A.....
S.boliviensis_ZFYA.....A.....A
P.cynocephalus_ZFYG..A.....A.....A.....
Felis_chaus_ZFY	.G.....G.....G.....
Bos_taurus_ZFYA.....A.....
BOWHEAD PROTEIN	P S E L K K H M R I H T G E K P Y Q C Q 160
Bowhead_ZFX	TACTGCGAATATAGGTCTGCAGACTCTTCTAACTGAAAACGCATGTAAAACTAAGCAT
Human_ZFXC.....
S.boliviensis_ZFXC.....
P.cynocephalus_ZFXC.....
Felis_chaus_ZFXG.....
Bos_taurus_ZFXC.....
Bowhead_ZFYT.....T.....T.....
Human_ZFYT.....A..A..A.....
S.boliviensis_ZFYT.....A..A..A.....
P.cynocephalus_ZFYT.....A..A..A.....
Felis_chaus_ZFYT.....G.....
Bos_taurus_ZFYT.....G.....
BOWHEAD PROTEIN	Y C E Y R S A D S S N L K T H V K T K H 180
P2-3EZ	
Bowhead_ZFX	AGTAAGAGATGCCATTCAAGTGTGACATTGTCTTCTGACTTTTCTCAGATACCAAAGAAGTGC
Human_ZFXG.....G.....
S.boliviensis_ZFXG.....G.....
P.cynocephalus_ZFXG.....G.....
Felis_chaus_ZFXC.....G.....
Bos_taurus_ZFXG..C.....
Bowhead_ZFY
Human_ZFY
S.boliviensis_ZFYC.....T.....
P.cynocephalus_ZFYT.....
Felis_chaus_ZFYC.....G.....
Bos_taurus_ZFYA..T..T.....C.....G..T..
BOWHEAD PROTEIN	S K E M P F K C D I C L L T F S D T K E V -- 201

Fig. 2 Continued

CY5 label degrades. Depending on the instrument used to read the fluorescent signal, it may also be possible to use a more stable fluorophore than CY5, thus eliminating this potential problem.

We tested sensitivity of the assay by performing quantitative PCR on a 10× dilution series of a single male *Tursiops truncatus* sample (in duplicate), ranging from 40 ng to 4 pg (five orders of magnitude, data not shown). Both the standard curve for the X (FAM) and the Y (CY5) had correlation coefficients of at least 0.98, and indicated an assay efficiency of approximately 100% (Smith *et al.* 2002). The Y chromosome product failed to amplify from both replicates at 4 pg/reaction, and the X chromosome product failed to amplify in one of the 40-pg reactions, but amplified both of the 4-pg reactions. In addition, 10-fold serial dilutions from four other male cetacean DNA samples (two *T. truncatus*, two *Balaenoptera musculus*) were used to test the sensitivity of the assay. The minimum quantities of DNA for which both of the products were detected were 9, 70, 110, and 490 pg (average = 170 pg).

Use of standard *Taq* polymerase instead of HotStartTaq was tested in several species (data not shown), and resulted in clear identification of sex with slightly reduced fluorescence signal. It is likely that regular *Taq* will work fine for most samples, but HotStartTaq may facilitate sexing from more degraded or low-concentration samples.

The 5' exonuclease assay correctly identified at least one individual of each sex from each of the 33 species tested

	1111122222	2333333344	4444445555	55566666	Number of clones
	1579900355	9011333801	3456891145	6680156	
	9291738616	0214567346	7681875887	3718704	
ZFX	CTTTGGGCAA	ACCACGCCAT	GCGCTCCATG	GCCCGCAT	4 (2)
BWf	10
BWm	10
GWfT.....	9
GWmT.....	13
SWfA.....A.....	2
SWmA.....A.....	9 (1)
PSfA.....G.....C.....A.....G.....	2 (1)
PSmA.....T.....G.....C.....A.....G.....	9 (2)
DSfA.....C.....A.....G.....	4
DSmA.....C.....A.....G.....	8 (1)
BLfT.....	11 (2)
BLmT.....	9 (1)
ESf	4
ESm	7
WSf	9 (1)
WSmT.....	5
HPfT.....	9
HPmT.....	3 (1)
FPf	14
FPm	
ZFY	TGCC.....	GTTGT..T.C	A.....G..	..TTT..	2
BWm	T.CC.....	..TTG..T..	A.A.....GC..	..GTTT..	8
GWm	TCCC..A..	GTTG.AGT.C	A.A.C..G..	..TTC..	6 (2)
SWm	TCCC.A..G	GTTG..T.C	A.A.C.....	..TTT..	6 (1)
PSm	TCCC.A..G	GTTG..T.C	A.A.C.....	..TTT..	9 (2)
DSm	T.CC..A.T.	GTTG..T.C	..AACT.G..	..TTT.G	2
BLm	T.CC..A.T.	GTTG..T.C	..AA.T.G..	..TTT.G	8 (2)
ESm	T.CC..A.T.	GTTG..T.C	..AA.T.G..	..TTT.G	5
WSm	T.CC..ATT.	..TTGT..T.C	..AA.T.G..	A.TTT.G	5 (1)
HPm	T.CC..ATT.	..TTGT..T.C	..AA.T.G..	A.TTT.G	10 (1)
FPm	T.CC..ATT.	..TTGT..T.C	..AA.T.G..	A.TTT.G	

Fig. 3 Comparison of ZFX and ZFY fixed and variable sites. f, female; m, male. Number in parentheses equals the number of clones that displayed replication errors. Gray highlighted positions denote eight fixed differences between ZFX and ZFY. ZFY variable sites 208, 251, 256, 336, 337, 567, and 664 display nonsynonymous substitutions. Positions 335 and 448 are variable sites in both the ZFX and ZFY. Species codes are as in Table 1.

	1111
	335584579
	382415860
	TRLNAKQVI
BWm ZFYE..
GWm ZFY	.H..S....
SWm ZFY	.H..S....
PSm ZFY	.H..S....
DSm ZFY	.H..S....
BLm ZFY	.HF.....S
ESm ZFY	.HF.....S
WSm ZFY	.HF.....S
HPm ZFY	.HF.....S
FPM ZFY	.HF.....S
Human ZFY	AH...R.I.
Human ZFX	A.....
Cetacean ZFX

Fig. 4 Amino acid variable site comparison between cetacean ZFX/ZFY and human ZFX/ZFY. The grey highlighted site indicates a fixed amino acid difference between cetaceans and humans on both the ZFX and ZFY. Sites 145 and 176 display fixed differences between the cetacean ZFY and human ZFY. Species codes are as in Table 1.

(Table 2). In total, 116 PCRs (not including no template controls, or NTC) were completed on 90 unique samples. Twenty of those (18 unique samples) failed to produce fluorescent signal, and presumably failed to amplify in the initial attempt. Eleven of those were re-assayed, and 10 were successfully assayed in a second attempt (usually with more dilute DNA and/or addition of BSA to minimize the effect of PCR inhibitors). Of the 86 successful assays, six initially contradicted field records. Two of those turned out to be simple data entry errors, and three were likely misclassifications by observers, as they were based on observation of whole animals, without inspection of genital slits or gonads. The sixth sample (3735, sexed as male by examination of gonads) was re-extracted from the

original tissue and sexed using this assay and an assay based on co-amplification of SRY and ZFX (Fain & Lemay 1995). The SRY assay failed again to work, but the 5' exonuclease assay confirmed that the sample is female, indicating a probable mix-up of samples prior to archival at the SWFSC.

One pair of bottlenose dolphin (*T. truncatus*) samples (4363, 4366) was used for assay optimization and as positive controls in several assays, so that both samples were sexed 10 times, and all agreed with the known sex of these individuals, indicating high replicability. In addition, 25 sperm whale samples that had previously been sexed using amplification of SRY (Richard *et al.* 1994) and keratin (NT Rubio – Cisneros *et al.*, CIBNOR, La Paz, Mexico, unpublished result) were re-assayed using this assay, and 24 were in agreement; the 25th did not amplify.

We tested the assay on human male DNA at four annealing temperatures (58, 60, 62, and 64 °C; data not shown). In all cases, the ZFX probe produced some signal, indicating that the probe is stable enough to be cleaved despite two mismatches with the human ZFX gene, and presumably is annealing to the ZFY gene, with which it has only one mismatch. Some signal despite probe mismatches can occur in 5' exonuclease assays, depending on their location (Morin *et al.* 1999; Smith *et al.* 2002), but could probably be overcome by use of higher annealing temperatures than tested here, or by use of 'minor groove binding probes', which make use of modified nucleotides to enhance probe specificity (Applied Biosystems). Detection of human ZFX will only likely prove a problem when significant human DNA contamination is likely, e.g. ancient DNA extracted from museum specimens, and can be identified through amplification plot slope (ASP) analysis or co-amplification of human specific loci (Wandeler *et al.* 2003).

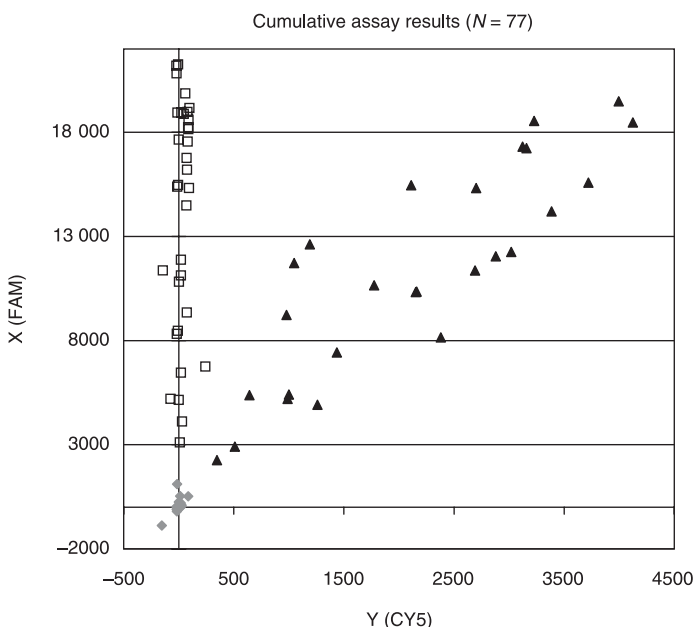


Fig. 5 Plot of normalized fluorescence values (dRn) for a subset of the samples tested with the 5' exonuclease assay, compiled from five different experiments. Fluorescence is measured as dRlast (baseline subtracted fluorescence reading). Variance in the relative position of the male signals (greater or lesser X to Y ratio) is due to variance in the PCR efficiency seen among species with primer site mismatches (e.g. site 152 is a mismatch for primer cetZFX_F1 on the ZFY gene in bowhead, sperm, pygmy sperm, and dwarf sperm whales).

Discussion

Sample selection

To determine with confidence the X-chromosomal and Y-chromosomal sequences of the zinc finger protein gene in cetaceans, known males and known females are critical. In nondimorphic cetacean species, examination of reproductive organs or external examination of the genital slit in relation to the anal slit (Jefferson *et al.* 1994) are the only two methods that can provide physical evidence of a specimen's sex, with the former providing an even higher level of confidence. At the time sequencing began, the SWFSC tissue archive contained 19 odontocete and two mysticete species that had at least one male and one female for which reproductive organ data had been recorded. Although this study aimed to represent the 14 extant cetacean families, paucity of gender information prevented the inclusion of the families Neobalaenidae, Balaenopteridae, Ziphiidae, Plantaniidae, Iniidae, Lipotidae, and Pontoporiidae in the initial sequencing of individuals of verified sex. For validation of the 5' exonuclease assay for sexing individuals, we expanded the sample set to include 33 of 87 cetacean species, in 9 of the 13 families (Table 2).

Human studies have demonstrated the ability to detect male fetal cells, using PCR amplification of the ZFY and/or SRY genes, not only in the maternal circulation (Liou *et al.* 1993; Lo *et al.* 1999) but also in skin lesions of pregnant females (Aractingi *et al.* 1998). As this phenomenon has yet to be studied in cetaceans, and to avoid the possibility of amplifying male fetal DNA instead of maternal DNA, resulting in possible errors in ZFX gene identification, pregnant females were avoided in sample selection. Although the female sperm whale was not necropsied to prove lack of pregnancy, genetic results indicate that the specimen was either not pregnant, was carrying a female fetus, or the Y copy of male fetal DNA was not detected. Amplification of female fetal DNA would not have interfered with identification of the ZFX gene.

Data accuracy and verification

Concordance of sequence data obtained from two independent rounds of PCR and cloning executed on five of the 20 samples also served to confirm data accuracy (Table 1). The consensus sequence for three other samples was determined from only two individual sequences but were each produced from a single PCR: BLm ZFY, SWm ZFX and PSm ZFX. BLm ZFY exhibited a unique haplotype, as similarly seen in BWm ZFY, but neither contributed any additional ZFY variable sites. SWm ZFX exhibited the same haplotype as SWf ZFX, a trend observed in the majority of the cetacean species examined. One of the two sequences obtained for PSm ZFX contained a nonsynonymous base

substitution at a nonvariable site. Because all the other ZFX sequences were observed to lack amino acid variability, the base substitution was regarded as a replication error. In addition, the other ZFX clone of PSm was identical to PSf ZFX.

The new 5' exonuclease assay presented here provides a simpler and potentially more reliable and accurate genetic method for sexing cetacean samples. We had high rates of amplification success, even from samples which had repeatedly failed to amplify the larger SRY/ZFX products (data not shown), and no examples of unreliable results (either results that changed when repeated, or which disagreed with other methods and which were determined to be false).

Conclusions

We have presented an analysis of the ZFX and ZFY genes from seven families of cetaceans, expanding substantially on what is currently known of these genes in Cetacea. We observed high levels of conservation within and between the loci, and little phylogenetic signal and no clear evidence of gene conversion within cetaceans, though ZFY was more polymorphic than ZFX and provided strong support for monophyly of the Physteridae and Kogidae, and of the Delphinidae, as seen with mtDNA (Arnason & Gullberg 1996).

This ZFX/ZFY 5' exonuclease assay has been found in this study to be a valid means to determine gender in 33 species representing nine of the 14 cetacean families, and most likely across the order Cetacea. This method can provide the secondary confirmation necessary for positive sex identification in marine mammal specimens, or a primary method where accurate field observation of gender is not possible. The assay is highly sensitive (down to at least 40 pg of DNA), rapid (approximately 3 h for PCR set-up and amplification of up to 96 samples), and unambiguous. Due to the small fragment size and use of a single pair of PCR primers to generate both the ZFX and ZFY products, the assay is likely to be highly accurate for degraded samples, and have potential application for ancient DNA.

Comparison of these new ZFX/ZFY sequences to published sequences of the same region and length reveals high levels of sequence conservation among several orders of mammals (Fig. 2). Literally thousands of ZFX/ZFY sequences from other mammals exist in the public databases. Although these primers have not been broadly tested for amplification of DNA from many mammalian orders, the original authors had demonstrated application to horse, sheep, goat, pig, mouse and rainbow trout (primer P2-3EZ, Aasen & Medrano 1990), and BLAST searches with these conserved primers show that they are highly homologous to sequences in other species (data not shown). It is likely that the primers used here will be applicable for

other mammalian orders for amplification of the same region, for characterization of conserved differences between sexes and subsequent development of broadly applicable 5' exonuclease sexing assays.

Finally, the instruments needed to detect the fluorescent signals during or after PCR (in PCR tubes) are now widely available in molecular and evolutionary biology laboratories, and with models and prices meant for the nonmedical laboratory. The additional uses for quantitative PCR and fluorescent product detection for sample quantification, genotyping (Morin *et al.* 1999, 2001) and expression analyses make this technology a welcome and versatile new tool in the molecular ecology toolbox.

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